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REVIEW PAPER

# Analytical methods applied for the characterization and the determination of bioactive compounds in coffee

**Magdalena Jeszka-Skowron · Agnieszka Zgoła-Grześkowiak · Tomasz Grześkowiak**

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**Abstract** Coffee, the one of the most popular beverages in the world, contains many bioactive compounds especially caffeine—the natural stimulant and chlorogenic acids with antioxidative properties. Other chemicals such as diterpenes may influence human serum lipids and protect from the risk of some types of cancer. All these compounds are widely determined in *Coffea arabica* and *robusta* green and roasted coffee beans, coffee brews and instant coffees to increase food quality standards. The most important analytical methods are reviewed, including these using high-performance liquid chromatography, ultraviolet spectrophotometry and voltammetry techniques, that have been applied to quantify the major bioactive compounds of coffee samples: phenols—inter alia chlorogenic acids and derivatives; methylxanthines—caffeine; trigonelline; nicotinic acid; diterpenes; and short-chain carboxylic acids. Usage of hyphenated techniques such as liquid chromatography–mass spectrometry and gas chromatography–mass spectrometry for identification and determination of coffee constituents is also presented.

**Keywords** Coffee · Chlorogenic acids · Caffeine · Trigonelline · Nicotinic acid · Diterpenes

## Introduction

Coffee is prepared from the roasted seeds (beans) derived from a bush of the genus *Coffea*. This drink was first prepared in Ethiopia and then in fifteenth century in Yemeni Sufi monasteries, from where it disseminated into the rest of the Middle East and northern Africa. The intense trade of the Venetian ships with the Middle East opened the doors of Europe to coffee, from where it was subsequently introduced to America [1].

Among about 500 species of coffee, only *Coffea arabica* and *Coffea canephora* (*robusta)* have significant position in production and export. These two species differ by appearance and origin and lastly but most importantly by its quality and flavor. Mild Arabica comes from upland and mountain areas of East Africa (Ethiopia) and Robusta from lowland of Central and West Africa.

The coffee consumption in emerging countries noted 21 % rise between 2009 and 2012. This corresponds to substantial increase in world coffee production which rose from 133.5 million (60-kg bags) in 2010/2011 crop year to 144.6 million in 2012/2013 [2]. There are numerous coffee producers, but Brazil and Vietnam reached the considerable production of coffee with 32.4 and 17.9 % share, respectively [3]. Among the most significant Brazilian coffee cultivars, *Coffea arabica* cv. Mundo Novo, *C. arabica* cv. Catuaı Vermelho, *C. arabica* cv. Bourbon and *Coffea canephora* cv. Conillon (Robusta) are the most popular blends around the world.

Traditionally, it was said that drinking coffee, especially in high amount, caused liver diseases, and therefore, its consumption should be low. But nowadays, the phytochemistry of coffee is known with about 1,000 described phytochemicals. For that reason, in last few decades biological effect of coffee brew on human organism has been widely analyzed and this beverage was named as functional food [4]. Thus, drinking coffee—in appropriate amount—has impact on the cardiovascular system and on the metabolism of carbohydrates and lipids. Opposing to previous

M. Jeszka-Skowron (✉) · A. Zgoła-Grześkowiak · T. Grześkowiak
Institute of Chemistry and Technical Electrochemistry, Poznan University of Technology, 60-965 Poznań, Poland
e-mail: magdalena.jeszka-skowron@put.poznan.pl

beliefs, the various forms of arterial cardiovascular disease, arrhythmia or heart insufficiency, seem to be unaffected by coffee intake. Coffee reduces the incidence of cancer, diabetes and liver disease, protects against Parkinson's disease and even reduces mortality risk [5, 6].

The most important bioactive compounds of coffee include the following: phenolic compounds (such as chlorogenic acids and derivatives), methylxanthines (caffeine, theophylline and theobromine), diterpenes, (including cafestol and kahweol), nicotinic acid (vitamin $B_3$) and its precursor trigonelline, magnesium and potassium [7]. This variability of the coffee chemical constituents should be determined using sensitive, precise and accurate analytical methods to examine the quality, aroma and properties of green and roasted coffee beans, instant coffees and coffee brew.

The traditional sample pre-treatment techniques used in food analysis are liquid–liquid extraction (LLE) and solid phase extraction (SPE) [8]. However, many other techniques are also used including drop-to-drop microextraction [9] or extraction using supercritical carbon dioxide [10]. The processed samples are then analyzed with the use of the advanced separation techniques, such as capillary electrophoresis (CE), high-performance liquid chromatography (HPLC) and ultra-high-pressure liquid chromatography (UHPLC) gas chromatography (GC) as well as with hyphenated techniques using mass spectrometry detection.

The aim of the review is to present and compare the analytical methods that have been applied to characterize and quantify the important bioactive compounds of coffee samples: phenols (chlorogenic acids and derivatives), methylxanthines (mainly caffeine), trigonelline, nicotinic acid, diterpenes and short-chain carboxylic acids.

## Determination of phenolic compounds

### Phenolic acids and lactones

The main groups of phenolics in coffee bean are chlorogenic acids (CGA) presented in Fig. 1 according to their chemical identity, number and position of acyl residues. This group of compounds includes the following: caffeoylquinic acids (CQA), with 3 isomers (3-, 4- and 5-CQA), dicaffeoylquinic acids (diCQA) with 3 isomers (3,4-diCQA; 3,5-diCQA; 4,5-diCQA), feruloylquinic acids (FQA) with 3 isomers (3-, 4- and 5-FQA), *p*-coumaroylquinic acids (pCoQA) with 3 isomers (3-, 4- and 5-pCoQA) and mixed diesters of caffeoylferuloylquinic acids (CFQA) [11, 12]. The first three compounds account for about 83 % of the total CGA in green beans [13]. Chlorogenic acids act as antioxidants [14] and showed hepatoprotective, hypoglycemic and antiviral activities.

The group of CGA is present in the coffee beans in high concentration and determines the quality of coffee and its flavor [15]. However, the total CGA content of green coffee beans may vary not only according to genetics—species but also to the degree of maturation, agricultural practices, climate and soil [16, 17]. For example CGA content found in different coffee species of wild *Coffea* genetic resources varies between 0.61 and 1.05 % for *Coffea* sp. Bakossito and 11.1–12.7 % dry basis for *Coffea* sp. N' koumbala [18].

To analyze these compounds in green coffee beans, the material must be crushed and frozen. It can be extracted with methanol/water mixture, filtered and analyzed directly using HPLC according to the official norm [19]. Many other procedures include sample clean-up. These include the following:

1. overnight extraction using 70 % (v/v) methanol/water mixture at 4 °C and then (after evaporation of methanol): LLE of water solution with different organic solvents [20] or filtration through a $C_{18}$ cartridge [21] or extraction on activated carbon [22] or treatment by Carrez reagents [23];
2. overnight extraction using 70 % (v/v) methanol/water mixture at 4 °C and then (without evaporation of methanol) treatment by Carrez reagents [15, 24];
3. 15 min extraction with methanol/water/acetic acid (30:67.5:2.5, v/v/v) with ascorbic acid (2 g/L) in an ultrasonic bath [25].

Furthermore, microwave-assisted extraction [26] and extraction using boiling water under elevated pressure [27] have been successfully developed. These two methods are advantageous because some of Carrez reagent products could react with CGA in the HPLC phase [28]. Table 1 shows the different methods of extraction process used for the determination of CGA.

The determination of wide range of phenolic compounds in green coffee beans in one analysis can be problematic. Therefore, the LC–MS system should be applied to improve selectivity. Fourteen CGA compounds—3-CQA, 4-CQA, 5-CQA, 3-FQA, 4-FQA, 5-FQA, 3-p-CoQA, 4-p-CoQA, 5-p-CoQA, 3,4-diCQA, 3,5-diCQA, 4,5-diCQA, 3,4-diFQA and 3,4-di-p-CoQA—and six CFQA isomers were identified in green *C. arabica* and *C. canephora* samples using LC–$MS^n$ system [29]. No lactones of chlorogenic acids were determined in green coffee samples, with the exception of a small amount of 3-FQL present in Mundo Novo cultivar. Also three caffeoylquinic acids CQA, three FQA, one pCoQA, three diCQA, three FCQA, four pCoCQA, three diFQA, six DCQA and six DFQA were found [25]. Moreover, three trans-cinnamic acids (caffeic, ferulic and dimethoxycinnamic), six cinnamoyl-amino acid conjugates (caffeoyl-*N*-tyrosine, p-coumaroyl-*N*-tyrosine, caffeoyl-*N*-tryptophan,

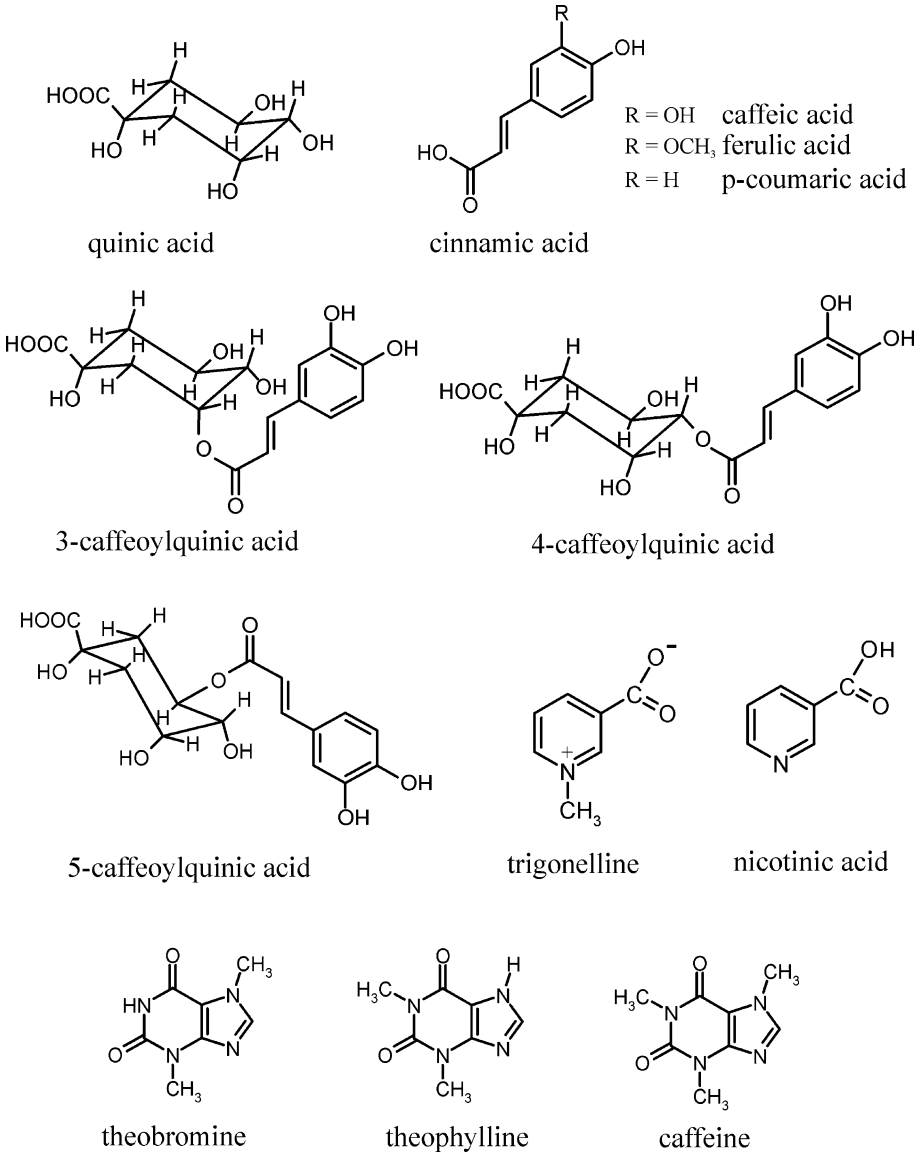


**Fig. 1** Chemical structures of chlorogenic acids and derivatives, caffeic acid, ferulic and *p*-coumaric acids, trigonelline, nicotinic acid, theobromine, theophylline and caffeine determined in coffee

p-coumaroyl-*N*-tryptophan, feruloyl-*N*-tryptophan, caffeoyl-*N*-phenylalanine) and three cinnamoyl glycosides (caffeoylhexose, dicaffeoylhexose and dimethoxycinnamoylhexose) were also determined in the coffee bean samples [25]. The highest level of phenolic compounds was determined in Robusta green coffee beans. Likewise, the liquid chromatography multi-stage spectrometric technique has been used to determine 3,4,5-triacylchlorogenic acids in coffee beans [29]. Kuhnert et al. [30] analyzed aqueous methanolic extracts by LC–ESI–TOF–MS. The procedure was successfully applied for analysis of 38 green bean coffee samples, which vary in terms of coffee variety and processing conditions.

CGA can also be analyzed using micellar electrokinetic chromatography (MEKC). In the proposed method, caffeoylquinic (chlorogenic) acid, caffeic and ferulic acid were determined in green coffee. A silica capillary with a solvent system that utilizes phosphate buffer and methanol was applied and the analysis lasted for only 9 min [31].

Besides the techniques allowing for separation of components, UV spectrometry analyses of CGAs were also developed [26, 32]. These methods are not so selective like the LC–MS procedures. However, they can be used for fast scanning giving basic information.

Chlorogenic acids of coffee brew are responsible for aroma and astringency. During roasting, a part of CGA is isomerized, a part is transformed into quinolactones due to dehydration and formation of an intramolecular bond, and a part is hydrolyzed and degraded into low molecular weight compounds or even reduced [12, 13, 16]. It depends on parameters of roasting process and coffee preparing [33–35].

Therefore, there are many analytical methods to identify and quantify these compounds in different coffee brews.

**Table 1** Extraction, identification and/or quantification of three main chlorogenic acids found in green coffee beans (Arabica and Robusta)

| Extraction method | Technique | Column | Eluent | Total CGA: 3,4 and 5-*O*-caffeoyl quinic acid (% db) | References |
|---|---|---|---|---|---|
| Extraction with water, then SPE on C18 cartridge | HPLC–UV | ODS Hypersil<br>5 µm, 200 × 4.6 mm | A: 15 % methanol in a citrate-hydrochloric acid buffer (pH 3)<br>B: 45 % methanol | CGA identification without quantitative analysis | [21] |
| Extraction with aqueous methanol and clarification with Carrez reagents | HPLC–UV | Superspher 100 RP 18<br>5 µm, 250 × 4 mm | A: 2 mM phosphoric acid (pH 2.7) and 5 % methanol<br>B: 2 mM phosphoric acid (pH 3.9) and 95 % methanol | *Coffea arabica* 4.1<br>*C. canephora* 11.3 | [56] |
| Extraction with aqueous methanol and clarification with Carrez reagents | LC–ESI–MS | Phenylhexyl<br>5 µm 150 × 3 mm | A: water/acetonitrile/acetic acid (980:20:5 v/v, pH 2.68)<br>B: acetonitrile/acetic acid (1,000:5 v/v) | CGA identification without quantitative analysis | [12] |
| Extraction with aqueous methanol and clarification with Carrez reagents | HPLC–UV | ODS-C18 Rexchrom<br>5 µm, 250 × 4.6 mm | 10 mM trisodium citrate/methanol (65:35 v/v, pH 2.5) | Brazil *Coffea arabica*<br>Rio Zona 7.02<br>Soft 5.78 | [15] |
| Extraction with methanol and water (4:6) | MEC<br>CE–UV | X-Terra MS C18<br>5 µm, 50 × 1.9 mm | SDS (70 mM) phosphate (17.6 mM) methanol (5 % v/v) buffer system, pH 2.5 | Brazil coffee beans<br>2.32–5.06 | [31] |
| Extraction with aqueous methanol and clarification with Carrez reagents | LC–ESI–MS | Magic C30<br>5 µm, 150 × 2 mm | A: 0.3 % formic acid<br>B: methanol | *C. arabica*, cv. Mundo Novo 5.08<br>*C. arabica* cv. Catuaı Vermelho 4.75<br>*C. canephora*, cv. Conillon 6.45 | [29] |
| Extraction with methanol/water/acetic acid containing ascorbic acid using sonication | HPLC–DAD–MS | Symmetry C18<br>5 µm, 250 × 4.6 mm | A: 0.2 % acetic acid<br>B: methanol | *C. arabica* ($n = 50$)<br>2.58–5.41<br>*C. robusta* (n = 57)<br>2.79–5.54 | [25] |
| Soxhlet extraction with 70 % methanol and clarification with Carrez reagents | LC–ESI–TOF–MS | Diphenyl<br>5 µm, 150 × 3 mm | A: water/formic acid (1,000:0.005 v/v)<br>B: methanol | CGA identification without quantitative analysis | [30] |
| Extraction with water (at 80 °C), 70 % methanol or 60 % isopropanol at pH 3.0, then addition of activated carbon for isolation | PLC-UV | Hypersil C18<br>5 µm, 250 × 4.6 mm | 70 % methanol | *Coffea arabica*<br>4.67–5.28 | [100] |

Most of them use chromatographic techniques (Table 2). Perrone et al. [29] found, apart from nineteen previously identified CGA and chlorogenic acid lactones, also such compounds as: 1-feruloylquinic acid, 1-feruloylquinic lactone and 3,4-diferuloylquinic acid in *C. arabica* and *C. canephora*, 3- and 4-p-coumaroylquinic lactones in *C. canephora* and 3,4-di-p-coumaroylquinic acid in *C. Arabica*.

The other procedure enabled simultaneous determination of CGA and derivatives using HPLC–DAD–$MS^n$. Seventeen chlorogenic acids, five chlorogenic acid lactones, two cinnamoyl-amino acid conjugates and two free cinnamic acids were analyzed in coffee brews [36]. Although CFQA isomers were previously found in roasted coffee [29, 37], it was the first study where each CFQA isomer was identified and quantified separately in roasted coffee. Latest research using LC–$MS^n$ system showed that also cinnamoylshikimate esters can be found in roasted coffee [38]. Jaiswal et al. were able to discriminate between the individual types of cinnamoylshikimate esters and chlorogenic acid lactones, and these compounds were assigned on the basis of $MS^3$ patterns of fragmentation [38].

Kaiser et al. [39] used high-speed countercurrent chromatography to isolate milligram amounts of lactones. They found the new structures of 3-*O*-caffeoyl-γ-quinide, 4-*O*-caffeoyl-muco-γ-quinide and 5-*O*-caffeoyl-epi-δ-quinide, and they confirmed them by 1D and 2D NMR spectroscopy including $^{13}C$ NMR data. It was shown that complex quinide mixtures could be separated by UHPLC in shorter time with the same or even better resolution compared with common HPLC analysis.

Apart from chromatographic techniques more widely used become electroanalytical methods, especially voltammetry due to its simplicity, fast response, low cost and satisfactory sensitivity have been established. The most popular techniques differential pulse voltammetry, square-wave voltammetry and adsorptive stripping voltammetry were successfully applied for analysis of chlorogenic acid in coffee brew [40–45]. These methods are based on glassy carbon electrode [45] or carbon paste electrode [41, 42] or Au electrode [43], or boron-doped diamond (BDD) electrode [44] with different modifiers such as multi-walled carbon nanotubes [45] or ionic liquid containing iridium nanoparticles and polyphenol oxidase [42], or molecular imprinted polymer [43]. However, it must be taken into consideration that these electroanalytical methods, although inexpensive, can only be used for basic analyses. Selective determination of a number of particular CQAs (contrary to LC–MS methods) is not possible.

### Isoflavones: daidzein, genistein and formononetin

Apart from phenolic acids and lactones and derivatives, coffee fruits and brew contain other important bioactive phenolics such as isoflavones, lignans, tannins and anthocyanins. Isoflavones such as daidzein, genistein or formononetin and lignans such as secoisolariciresinol, matairesinol, pinoresinol or lariciresinol belong to the group of phytoestrogens which are used to protect from hypercholesterolemia, carcinogenesis and osteoporosis, and they relieve menopausal symptoms [46].

Isoflavones and other phytoestrogens of coffee samples are usually determined with the use of HPLC [47] or gas chromatography–mass spectrometry (GC–MS) [48, 49] or using automatic SPE and LC–MS/MS [50]. To free the aglicones of isoflavones from glycosides or glycoside esters and quantify them, acid hydrolysis [47] or enzymatic procedure [51] was applied. Thompson et al. [49] analyzed the commercial coffee samples for isoflavones (genistein, daidzein, glycitein, formononetin), lignans (secoisolariciresinol, matairesinol, pinoresinol, lariciresinol) and coumestan (coumestrol) with the use of GC–MS. Instant coffee contain 1,000-fold higher level of phytoestrogens than coffee brew [52].

## Determination of caffeine and other methylxanthines

Caffeine (1,3,7-trimethylxanthine) is the major alkaloid present in green coffee beans which also can be found in considerable concentration in roasted coffee [36, 53, 54]. Because of its stimulating effect on the central nervous system, it is also the most widely known constituent of coffee [55]. However, it is not associated by most people with taste of coffee brew although it contributes to the brew bitterness [15, 56].

Concentration of caffeine in coffee beans depends mainly on its species. Green beans of *Coffea arabica* contain between 0.7 and 1.6 % caffeine and of *Coffea canephora* between 1.5 and 4.0 % [54, 56]. Two other methylxanthines are also reported in coffee brews, i.e., theobromine (3,7-dimethylxanthine) and theophylline (1,3-dimethylxanthine). However, these methylxanthines can be found in coffee in considerably lower amounts than caffeine and are rarely analyzed [36].

Caffeine is determined in coffee samples usually with the use of HPLC. Sample preparation before this analysis is usually very simple and contains only filtration and dilution steps [54, 57, 58]. Some methods include clarification with Carrez reagents [15, 36, 53] or with lead acetate [59]. Alternatively, sample can be cleaned up by water extraction of powdered coffee beans mixed with magnesium oxide [56] or using solid phase extraction [27, 60]. The samples prepared according to one of these procedures were analyzed in a reverse phase elution mode with octadecylsilica-packed column. Caffeine was detected with the ultraviolet absorbance detector at 270–280 nm [11, 15, 27, 36, 53, 54, 56–58, 60]. Alternatively, HPLC with mass spectrometry

**Table 2** Extraction, identification and/or quantification of chlorogenic acids in roasted coffee brew and extracts

| Extraction method | Technique | Column | Eluent | Analyzed compounds | References |
|---|---|---|---|---|---|
| Extraction with aqueous methanol and clarification with Carrez reagents | LC–ESI–MS | Magic C30<br>5 µm, 150 × 2 mm | A: 0.3 % formic acid<br>B: methanol | 3-CQA; 4-CQA; 5-CQA; 3-FQA; 4-FQA; 5-FQA; 3-p-CoQA; 4-p-CoQA; 5-p-CoQA; 3,4-diCQA; 3,5-diCQA; 4,5-diCQA; 3,4-diFQA; CFQA; 3-CQL; 4-CQL; 3-FQL; 4-FQL; 3-p-CoQL; 4-p-CoQL; 3,4-diCQL | [29] |
| Extraction with aqueous methanol and sonication for 10 min, followed by shaking at 300 rpm for 30 min | HPLC–UV-ESI–MS | Symmetry ShieldTM-C18<br>5 µm, 250 × 4.6 × mm | A: 0.4 % formic acid<br>B: acetonitrile | 3-CQA; 4-CQA;5-CQA; 3-FQA; 4-FQA; 5-FQA; 3,4-diCQA; 3,5-diCQA; 4,5-diCQA; 3-CQL;4-CQL; 5-CQL; 3-FQL; 3-FQL; 5-FQL | [101] |
| Extraction with 40 % aqueous methanol and clarification with Carrez reagents | HPLC–UV and LC–ESI–MS | ODS-C18 Rexchrom<br>5 µm, 250 × 4.6 nm | A: 10 mM citric acid<br>B: methanol | 3-CQA; 4-CQA; 5-CQA; 3-FQA; 4-FQA + 5-FQA; 3,4-diCQA; 3,5-diCQA; 4,5-diCQA | [102] |
| Extraction of 2 g coffee with 20 ml of water at 100 °C for 15 min with occasional stirring | HPLC–UV | Pinnacle II C-18<br>5 µm, 250 × 4.6 mm | A: 3 % formic acid<br>B: methanol | Sum of 3-CQA, 4-CQA and 5-CQA | [54] |
| Soxhlet extraction using 70 % aqueous methanol for 5 h and clarification with Carrez reagents | LC–ESI–$MS^n$<br>(FT–ICR mass spectrometry) | Diphenyl<br>5 µm, 150 × 3 mm | A: 0.5 % formic acid<br>B: methanol | C1-acylated CGAs arising from acyl migration reactions;<br>dehydrated CGA derivatives including lactones and shikimic acid derivatives and epimerization products | [103] |
| Extraction of 5 g roasted ground coffee or 1 g soluble coffee with 50 mL water and clarification with Carrez reagents | HPLC–DAD–ESI–$MS^n$ | ODS-C18 Shim-pack<br>5 µm, 250 × 4.6 mm | A: 80 % 10 mM citric acid (pH 2.5) and 20 % methanol<br>B: methanol | CQA, FQA, p-CoQA, diCQA, CFQA, chlorogenic acid lactone subgroups (CQL, FQL), cinnamoyl-amino acid conjugates (CTrp, p-CoTrp) and free cinnamic acids (caffeic acid, p-coumaric acid) | [36] |
| Extraction with water and clarification with Carrez reagents | HPLC–UV | Eclipse plus C18<br>1.8 µm, 100 × 2.1 mm | A: 0.1 % formic acid<br>B: acetonitrile(with 0.1 % formic acid) | 5-CQA and 3-CQA | [53] |

was used for determination of caffeine [59] or theobromine and theophylline [36].

Other chromatographic techniques are rarely used for the determination of caffeine in coffee samples. Examples include GC–MS [9] and MEKC with ultraviolet absorbance detection [61]. Relatively fast and simple sample preparation was used in both these studies. Drop-to-drop microextraction from water sample to chloroform was used before GC analysis [9]. Chloroform extraction from beans, evaporation and reconstitution into water was used before MEKC analysis [61].

Non-chromatographic methods applied for the determination of caffeine included use of several techniques. Direct analysis in real-time ionization (DART)—high-resolution mass spectrometry—was used as a fast and selective technique. The coffee brew sample for this analysis can be placed directly in the DART ion source, and time of flight mass spectrometer enables high-resolution measurement of obtained mass spectra, i.e., high selectivity. Variations of signal due to matrix effect and ion source instability were compensated by addition of isotopically labeled caffeine standard [62].

Caffeine was also determined with the use of Fourier transform infrared (FTIR) spectroscopy in combination with attenuated total reflectance (ATR) techniques. In the proposed method, a chloroform extract of caffeine from coffee brew was evaporated on a zinc selenide crystal. Caffeine signal was monitored at 1,655 $cm^{-1}$ [63]. The method is faster and relatively simple. However, combination of ATR with FTIR is not a popular analytical technique. Moreover, only one compound was determined in the presented procedure.

Other methods used for the determination of caffeine in coffee include UV spectrometry [54, 64, 65], voltammetry [44, 66], gravimetric analysis [54] and paper substrate phosphorescence [67]. As direct measurement of caffeine UV absorbance in water extract of coffee is impossible because of high matrix effects, Balay et al. [64] proposed usage of dichloromethane for its extraction before UV measurement. Hečimovič et al. [54] used two different procedures for sample clean-up before UV analysis. In the first one, lead acetate was used for clarification, and in the second one extraction with benzene and back extraction to 5 N $H_2SO_4$ were applied. The results obtained with these methods were, however, considerably lower than with the reference HPLC method [54]. On the other hand, the use of a gravimetric method proposed in the same paper resulted in higher agreement with the HPLC method. In this procedure, coffee brew was clarified with lead acetate, extracted with chloroform and washed with KOH solution. Caffeine was determined gravimetrically from evaporated chloroform extract [54]. Another approach to UV analysis was proposed by Salinas-Vargas and Cañizares-Macías who used complicated sample preparation before the measurement [65]. The coffee samples were clarified with the Carrez reagents and then cleaned up using on-line SPE procedure with octadecylsilica cartridge. Satisfactory recoveries were obtained in different samples of green and roasted coffee beans.

Caffeine can also be determined successfully with voltammetric methods. Yardim et al. [44] used a BDD electrode for simultaneous determination of caffeine and chlorogenic acid in coffee. As fouling occurred at the BDD electrode, anodic cleaning in 0.5 M $H_2SO_4$ was used. It enabled successful determination of caffeine and chlorogenic acid with limits of detection 0.55 and 1.26 μM, respectively [44]. Aklilu et al. [66] used a carbon paste electrode for determination of caffeine. Problems with high background in its determination led to high limit of detection which was solved by modification of the electrode. The 1,4-benzoquinone modified carbon paste electrode enabled indirect voltammetric determination of caffeine in coffee extracts with limit of detection 0.3 μM [66]. Mersal used a pseudo-carbon paste electrode (made from 65 % graphite powder and 35 % paraffin) in both cyclic voltammetry and square-wave voltammetric measurements of caffeine [68]. Among these two techniques, square-wave voltammetric was found better for the determination of caffeine as it led to a well-formed oxidation peak. The presented voltammetric methods are fast and inexpensive. However, not many compounds can be determined using these procedures.

Finally, an example of a rarely used analytical technique must be mentioned. The paper substrate room temperature phosphorescence was used for analysis of caffeine, theobromine and theophylline in selected products. In the developed method, a sample was introduced onto the filter paper strip together with KI/$CH_3Na$ solution used for signal enhancement. Phosphorescence of the sample spot was measured with a fluorescence spectrometer. The limits of detection were from 0.8 to 1.8 ng per spot. However, the method was not selective, as each of the three methylxanthines had analytical signal at the same region of wavelengths. Therefore, caffeine was determined as main constituent in coffee and tea, theobromine in chocolate and theophylline in tablets containing this active pharmaceutical ingredient [67].

## Determination of trigonelline and nicotinic acid

Trigonelline (*N*-methyl nicotinic acid) (Fig. 1) is a nitrogenous compound, a pyridine alkaloid that is derived from the methylation of the nitrogen atom of nicotinic acid (niacin). Trigonelline is the second main alkaloid found in green coffee beans. During the roasting process, trigonelline suffers severe thermal degradation generating a series

of volatile compounds responsible for flavor formation and aroma production and it is used as roasting-level discriminator in both Arabica and Robusta coffees [56, 59, 69]. The compounds, such as pyridine and pyrrole derivatives, nicotinic acid, methyl ester of nicotinic acid and above 20 different compounds, are formed [70, 71]. Trigonelline is also degraded by decarboxylation generating the *N*-methylpyridinium cation, the inductor of enzyme systems involved in detoxification of xenobiotics [72], activator of the Nrf2/ARE pathway, inducing cellular defense mechanisms [73, 74] and novel phytoestrogen [75].

HPLC is the main technique used for the determination of trigonelline and nicotinic acid in coffee beans. Several examples of these methods are presented in Table 3. Nevertheless, gas chromatography was also used for analysis of trigonelline [70].

The contents of trigonelline and nicotinic acid in coffee brews may be highly influenced by coffee species, variety, geographical origin and roasting conditions [56, 58]. Fast LC–MS method (6 min of analysis) simultaneously determined caffeine, trigonelline, nicotinic acid and sucrose [59]. This method showed appropriate recoveries and repeat abilities (RSD <5 %) for all analytes in the matrix. The limit of detection (LOD), calculated on the basis of signal-to-noise ratios of 3:1, was 11.9, 36.4, 18.5 and 5.0 ng $mL^{-1}$ for caffeine, trigonelline, nicotinic acid and sucrose, respectively. The content of trigonelline in coffee beans of Arabica was slightly higher than in Robusta (1.03 vs. 0.9 % on the dry basis). Nicotinic acid in extracts of regular commercial ground roasted coffee, decaffeinated and instant coffees was determined. There was no difference in its level between these beverages [59]. Similar results were found by Liu et al. [76] who developed the separation of trigonelline, nicotinic acid and caffeine by HPLC using two chromatographic columns in series.

Quantitation of trigonelline and *N*-methylpyridinium in roasted coffee was developed using a HILIC–LC–MS/MS method [77]. This investigation showed that the formation and degradation of these coffee bioactive compounds had influence on color and aroma.

Besides chromatographic techniques, the proton nuclear magnetic resonance technique ($^1$H NMR) was applied [78–80]. $^1$H NMR spectrum at the 7.5–10.0 ppm range for the quantitative and simultaneous determination of caffeine, formic acid, trigonelline and 5-(hydroxymethyl)-2-furaldehyde was obtained [80]. The time of analysis was 20 min with no need of any previous derivatization or treatment.

## Determination of diterpenes

Cafestol and kahweol as anti-nutritional factors are unique chemicals to coffee beans and brews. These compounds and coffee drinking are positively correlated with higher level of serum cholesterol lipids, especially in patients with hyperlipidemia [81, 82]. Latest research showed that they reduce the risk of colorectal cancer [83].

Analysis of diterpenes can be easily performed with the use of GC–FID. Soxhlet extraction can be used to extract these compounds from the beans [84]. Further treatment includes saponification which is also used in preparation of coffee extract samples, and then, the samples are silylated [84, 85]. Chartier et al. [86] proposed a simplified procedure with automatic transesterification of the esters of diterpenes in a flow reactor. Diterpenes from the reactor were further cleaned up and injected into GC-FID without silylation.

HPLC is the alternative technique which can be used for the determination of diterpenes. Gross et al. used SPE and saponification for coffee sample preparation and analyzed diterpenes with HPLC–UV at 230 nm for cafestol and 290 nm for kahweol [87]. Sridevi et al. [88] used a 6-h-long Soxhlet extraction for powdered coffee beans. The free diterpenes from unsaponified extracts or brews were determined with HPLV–UV at the above-mentioned wavelengths. On the other hand, Araujo and Sandi developed green chemistry method of extraction using supercritical carbon dioxide before sample saponification and analysis with the use of HPLC–UV at 220 nm [10]. Use of mass spectrometry for analyte detection is another option possible in HPLC analysis, which gives additional selectivity. Oigman et al. [89] analyzed kahweol and cafestol using LC-HRMS after microwave methanolysis of coffee samples. Latest research showed that for the determination of diterpenes in green coffee samples the 10-min-long microwave-assisted extraction was considerably less time consuming than the 4-h-long traditional Soxhlet extraction, while amount of extracted diterpenes was similar [90]. Other modern extraction method—used for spent coffee grounds—was supercritical fluid extraction. This optimized extraction method led to diterpenes concentrations from 2 to 4 times higher than using Soxhlet apparatus [91]. Scharnhop and Winterhalter employed high-speed countercurrent chromatography for the isolation and purification of different diterpenes (i.e., kahweol, cafestol, 16-*O*-methylkahweol, 16-*O*-methylcafestol, dehydrokahweol, and dehydrocafestol) from *Coffea arabica* and *Coffea canephora* var. *robusta* [92]. The solvent systems consisted of hexane–ethyl acetate–ethanol–water mixtures. Identity and purity of the isolated compounds were confirmed by HPLC–DAD and LC–$MS^n$ as well as NMR measurements. Another NMR method developed for the analysis of green coffee beans required the 4-h-long Soxhlet extraction with pentane. After drying and evaporation of pentane, obtained coffee oil was subjected to NMR analysis which enabled clear discrimination between Arabica and Robusta [93].

**Table 3** Determination of trigonelline with other compounds in coffee samples using different chromatographic techniques

| Extraction method | Technique | Column | Eluent | Analyzed compounds | References |
|---|---|---|---|---|---|
| Extraction of 0.5 g coffee with hot water (80 °C) in a water bath, with shaking for 15 min | HPGF–UV | TSK G-3,000 SW HPGF 300 × 8 mm | Water | Trigonelline, caffeine, chlorogenic acid | [104] |
| Extraction of 2 g coffee with 100 ml boiling water | HPLC–DAD | Spherisorb S5 ODS2 10 µm, 250 × 4.6 mm | A: phosphate buffer 0.1 M (pH 4.0) B: methanol | Trigonelline, caffeine, nicotinic acid | [58] |
| 50 mg coffee, 500 mg of magnesium oxide with 25 ml of water were heated for 20 min at 105 °C in an autoclave | HPLC–UV | Superspher 100 RP 18 column 5 µm, 250 × 4 mm | A: solution of triethylamine and acetic acid (pH 5.3) B: methanol | Trigonelline, caffeine | [55] |
| Extraction with hot water and clarification with 60 % lead acetate | HPLC–UV | ODS C-18 Merck | 40 % methanol for caffeine and 5 % methanol for trigonelline | Trigonelline, caffeine | [15] |
| 0.2 g of ground coffee (or 0.1 g instant coffee) was suspended in 60 mL of boiling water and shaken for 15 min at 300 rpm | LC–ESI–MS | Spherisorb S5 ODS2 5 µm, 150 × 2.0 mm | A: 0.3 % formic acid B: methanol | Trigonelline, caffeine, nicotinic acid, caffeine, sucrose | [29] |
| Extraction with 95 °C water and clarification with 20 % lead acetate | HPLC–UV | ODS C-18 Merck | 40 % methanol for caffeine and 5 % methanol for trigonelline | Trigonelline, caffeine | [102] |
| 1 g of coffee and magnesium oxide were homogenized in water (100 mL) and placed in a water bath at 90 °C for 20 min, with continuous stirring | HPLC–UV-VIS | Lichrosorb 100 RP-18 5 µm, 250 × 4 mm | 20 mM phosphate buffer (pH 4.3) and acetonitrile (9:1) | Trigonelline, caffeine | [69] |
| Microwave-assisted extraction at 120 °C, 300 W in 3 min | HPLC–UV | Polyaromatic hydrocarbon (PAH) C18 5 µm, 250 × 4.6 mm and Bondapak $NH_2$ 5 µm, 300 × 3.9 mm | 0.02 M phosphoric acid/methanol (70:30, v/v) | Trigonelline, caffeine, nicotinic acid | [76] |
| 200 mL of water clarification with 20 % lead acetate | HPLC–DAD-$MS^n$ | ODS-C18-Shim-pack 5 µm, 250 mm × 4.6 mm | A: 0.5 % acetic acid (pH 3) B: methanol | Trigonelline, nicotinic acid, 5-HMF, theobromine, theophylline | [36] |
| 50 mL of water at 95 °C, stirring for 5 min | HPLC–UV | Spherisorb ODS2 5 µm, 250 × 4.6 | A: 5 % acetic acid B: acetonitrile | Trigonelline, caffeine, furfural, 5-CQA, HMF | [35] |

Coffee beans contain 0.15–0.37 % (Robusta) and 0.27–0.67 % (Arabica) d.m. of cafestol. Similarly kahweol levels are 0.1 % d.m. and 0.11–0.35 % d.m. in Robusta and Arabica beans, respectively [94]. Cafestol and kahweol levels in brews such as espresso, Turkish-style and French press coffee were significantly higher than in filtered or instant coffee [87]. Similarly higher roasting temperatures and prolonged roasting times had influence on diterpenes profiles in roasted beans [88, 95]. Cafestol and kahweol were degraded to dehydrocafestol and dehydrokahweol after 8 min of roasting process [95].

### Determination of short-chain carboxylic acids and inorganic ions

Simple aliphatic carboxylic acids and inorganic acids are rarely analyzed in coffee although their acidity contributes to sourness of coffee brew and therefore to its taste. In fact, a major taste of high-quality Arabica coffee is sourness [60, 96]. Amount of acids in green coffee beans changes during their maturation. In the mature beans, mainly citric and malic acids were found at about 1.5 and 0.4 %, respectively. Among inorganic ions, phosphates dominated. They were found at concentration 0.1 % [97]. The roasting process, however, leads to changes in amounts of simple aliphatic carboxylic acids. Concentrations of citric and malic acids slightly decrease. In the same time, considerable increase in formic, acetic, glycolic and lactic acids can be observed, while phosphates are little affected by the roasting process [98]. Therefore, analysis of these compounds is so important.

The aliphatic carboxylic acids and phosphates can be analyzed using different analytical techniques. Verardo et al. [98] used gas chromatography with mass spectrometric detection. The acids were derivatized before analysis with diazobutane (used for low molecular weight (C1–C3) acids) or with diazomethane (used for other acids). The procedure was successfully applied for analysis of different coffee types and for analysis of changes in coffee caused by the roasting process [98]. Bähre and Maier also applied gas chromatography with mass spectrometric detection for analysis of carboxylic acids in coffee [99]. Samples were cleaned up before analysis using electrophoresis, and acids were derivatized with trimethylchlorosilane. A number of acids were identified including some that were not reported in coffee samples before [99].

Electrophoretic techniques can be used not only for sample clean-up. Capillary electrophoresis was successfully applied for the determination of short-chain organic acids in coffee. No derivatization was needed, and acids were analyzed using UV detector at 200 nm. The developed method was applied for determination of almost twenty different acids [96].

Simple aliphatic carboxylic acids were also analyzed using HPLC. Different columns and elution modes were used. Rogers et al. [97] applied high-performance anion exchange chromatography coupled to pulsed electrochemical detection. A number of coffee constituent were analyzed using different chromatographic columns. Carboxylic acids and inorganic anions were determined on an AS11 column (containing polymeric resin with alkanol quaternary ammonium functional groups) in a gradient of sodium hydroxide solution [97]. Rodrigues et al. [60] analyzed short-chain organic acids using HPLC in a reversed phase elution mode. Samples were cleaned up using a strong anion exchange solid phase extraction column. Then, they were analyzed on an octadecylsilica column using 25 mM pH 2.4 phosphate buffer with 1 % methanol for elution. Signal was monitored using UV detection at 210 nm. The developed method was applied for the determination of acids in a number of coffee samples from 10 different geographical origins [60].

## Conclusions

Chlorogenic acids and derivatives, caffeine and trigonelline seem to be the most popular compounds determined in green coffee beans, coffee brews and instant coffees. For standardized analysis of these compounds, HPLC–UV technique appears to be the most appropriate although faster separations are achieved using UHPLC. The use of the LC–MS techniques enables to measure entire range of chlorogenic acids and derivatives and can also be used for identification of unknown constituents.

**Conflict of interest** None.

**Compliance with Ethics Requirements** This article does not contain any studies with human or animal subjects.



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